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Structure of the 14-3-3 ζ –LKB1 fusion protein provides insight into a novel ligand-binding mode of 14-3-3

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The 14-3-3 proteins are a family of highly conserved proteins that play key roles in many cellular processes. The tumour suppressor LKB1 regulates cell polarity, cell growth and energy metabolism. 14-3-3 proteins bind to LKB1 and suppress its functions. Previously, preliminary crystallographic data for the 14-3-3 ζ –LKB1 fusion protein have been reported. Here, the crystal structure of this fusion protein was solved and a novel potential binding mode of 14-3-3 to its ligands was found.

1. Introduction

The 14-3-3 proteins are a family of highly conserved proteins that are widely expressed in all eukaryotic cells. 14-3-3 proteins have multiple important roles in a variety of cellular processes such as signal transduction, transcriptional regulation, cell growth, proliferation, apoptosis and membrane trafficking (Gardino & Yaffe, 2011; Fu *et al.*, 2000; Freeman & Morrison, 2011; Kleppe *et al.*, 2011). Seven 14-3-3 isoforms have been identified in mammals, named 14-3-3 β , 14-3-3 ϵ , 14-3-3 η , 14-3-3 γ , 14-3-3 τ , 14-3-3 ζ and 14-3-3 σ (Ichimura *et al.*, 1988; Martin *et al.*, 1993). All 14-3-3 proteins show a high similarity in sequence and structure, forming a clamp-shaped dimer with each subunit consisting of a highly conserved ligand-binding groove created by nine antiparallel α -helices (Obsil & Obsilova, 2011).

14-3-3 proteins were the first class of proteins that were discovered to be specific phosphoserine (pS)/phosphothreonine (pT) binding proteins. The target proteins usually share one of two common binding motifs: RSXpS/pTXP (mode I) or RXXXpS/pTXP (mode II) (where X represents any residue; Obsil & Obsilova, 2011; Rittinger *et al.*, 1999; Yaffe *et al.*, 1997). In these two binding modes, the phosphorylated residue pS/pT of the target protein is recognized by a positively charged pocket within the ligand-binding groove of the 14-3-3 protein. An arginine residue at the pS/pT –3 position in the mode I motif forms a salt bridge with acidic residues or a hydrophobic interaction with the aliphatic part of residues in its vicinity from the 14-3-3 protein, while an arginine at the pS/pT –4 position in the mode II motif presumably stabilizes the phosphopeptide conformation by forming an intramolecular ion pair with the phosphate of pS/pT (Rittinger *et al.*, 1999). A proline residue at the pS/pT +2 position in both the mode I and II motifs causes a turn of the peptide away from the binding groove of 14-3-3, presumably to prevent

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steric hindrance. In addition to the two canonical phosphorylated ligand-binding modes, protein C-termini binding by 14-3-3 proteins (mode III) has been identified and the consensus sequence is pS/pTX₁₋₂-COOH (Coblitz *et al.*, 2005, 2006; Würtele *et al.*, 2003; Ganguly *et al.*, 2005). 14-3-3 proteins can also bind unphosphorylated peptides such as peptide R18 and a peptide from exoenzyme S (Petosa *et al.*, 1998; Ottmann, Yasmin *et al.*, 2007; Yang *et al.*, 2006). Both of the two peptides have an amphipathic sequence which recognizes the ligand-binding groove in 14-3-3 proteins *via* hydrophilic and hydrophobic interactions.

The functions of 14-3-3 proteins are diverse and can be generally classified into three main types: (i) changing the conformation of a target protein, (ii) physically occluding sequence-specific or structural features of a target protein and (iii) acting as a scaffold to connect anchor proteins with one another (Bridges & Moorhead, 2004).

LKB1 is a key serine/threonine protein kinase that is ubiquitously expressed in adult and foetal tissues and its mutations play a causal role in Peutz–Jeghers syndrome, an autosomal dominant genetic condition that leads to a predisposition to a wide spectrum of benign or malignant tumours (Hemminki *et al.*, 1998; Jenne *et al.*, 1998). LKB1 functions as a tumour suppressor by regulating cell polarity, advancing cell differentiation and controlling cell division and energy metabolism (Ji *et al.*, 2007; Makowski & Hayes, 2008; Alessi *et al.*, 2006). LKB1 is activated by forming a heterotrimer with the scaffold protein MO25 and the pseudokinase STRAD and subsequently translocating to the cytoplasm (Boudeau *et al.*, 2004; Zeqiraj *et al.*, 2009). The canonical targets of LKB1 are AMP-activated protein kinase (AMPK), a master regulator of energy homeostasis and cell growth, and its related kinases (Woods *et al.*, 2003). The LKB1–AMPK signalling pathway regulates cell growth and metabolism *via* various downstream effectors (Hardie *et al.*, 2012).

A recent study demonstrated that 14-3-3 can interact with LKB1 by recognizing its phosphorylated Thr336 (Bai *et al.*, 2012). Through this interaction, 14-3-3 inhibits the binding of LKB1 to its substrates and thus suppresses its function. Moreover, the complex of 14-3-3 and LKB1 can regulate the activity and localization of QSK and SIK (Al-Hakim *et al.*, 2005). Previously, we reported preliminary crystallographic data for the fusion protein 14-3-3 ζ –LKB1 (Ding *et al.*, 2013). Here, we solved the crystal structure of this fusion protein and found a novel binding mode of 14-3-3 to its ligand.

2. Materials and methods

2.1. Crystallization and data collection

The procedure for the cloning, purification and crystallization of the fusion protein 14-3-3 ζ –LKB1, which contains sequences for 14-3-3 ζ (amino acids 1–230), a linker GSSGGS and LKB1 (amino acids 333–RSMTVVPY-340) with a phosphomimetic mutation T336E, was performed as reported previously (Ding *et al.*, 2013). The crystals were grown from conditions consisting of 1.4 M Li₂SO₄, 0.1 M bis-tris propane

Table 1

X-ray diffraction data collection and refinement for 14-3-3 ζ –LKB1.

Values in parentheses are for the highest resolution shell.

Data collection	
Wavelength (Å)	0.9793
Space group	<i>R</i> 32
<i>a</i> , <i>b</i> , <i>c</i> (Å)	130.262, 130.262, 264.960
α , β , γ (°)	90.0, 90.0, 120.0
Resolution (Å)	50.0–2.90 (2.95–2.90)
Total reflections	213600
Unique reflections	19507
<i>R</i> _{merge}	0.071 (0.922)
$\langle I/\sigma(I) \rangle$	43.0 (3.2)
Completeness (%)	100.0 (100.0)
Multiplicity	10.9 (11.3)
Wilson <i>B</i> factor (Å ²)	84.9
Refinement	
<i>R</i> _{work}	0.191
<i>R</i> _{free}	0.245
No. of atoms	
Total	3754
Protein	3700
Water	8
Ligand	46
R.m.s.d., bonds (Å)	0.009
R.m.s.d., angles (°)	1.190
Ramachandran plot	
Favoured (%)	100
Outliers (%)	0
Average <i>B</i> factor (Å ²)	83.3

pH 7.0. Diffraction data were collected on beamline BL17U at Shanghai Synchrotron Radiation Facility (SSRF), People's Republic of China from a single crystal using 1° oscillations with a crystal-to-detector distance of 400 mm. Diffraction data were processed using *HKL-2000* (Otwinowski & Minor, 1997). A complete data set was collected and the diffraction resolution was 2.9 Å.

2.2. Structure determination and refinement

The structure of the 14-3-3 ζ –LKB1 fusion protein was solved by molecular replacement with *Phaser-MR* in *PHENIX* using the 14-3-3 ζ structure (PDB entry 1a4o; Liu *et al.*, 1995) as a search model (McCoy *et al.*, 2007; Adams *et al.*, 2010). The model was completed using iterative cycles of model building with *Coot* and refinement with *phenix.refine* in *PHENIX* (Afonine *et al.*, 2012; Emsley *et al.*, 2010). In refinement, 10% of the reflections were selected for the calculation of *R*_{free}. The stereochemical quality of the final model was validated using *PROCHECK* (Laskowski *et al.*, 1993).

2.3. Structure deposition

The coordinates and structure factors for the crystal structure of the 14-3-3 ζ –LKB1 fusion protein were deposited in the Worldwide Protein Data Bank (wwPDB) under accession code 4zdr.

3. Results and discussion

The structure of the 14-3-3 ζ –LKB1 fusion protein was determined by molecular replacement using the apo 14-3-3 ζ structure (PDB entry 1a4o; Liu *et al.*, 1995) as a search model.

The data-collection and refinement statistics are shown in Table 1. The structure was refined with an R_{work} of 0.191 and an R_{free} of 0.245. In the asymmetric unit, there are two copies of the 14-3-3 ζ -LKB1 protein, in both of which nearly the whole sequence of 14-3-3 ζ (1–230) was modelled, with the exception of several residues in flexible loop regions (Fig. 1*a*). In one copy of the 14-3-3 ζ -LKB1 protein only electron density for the first and last residues of the linker region and the first four residues (amino acids 333-RSME-336) of LKB1 is clear and electron density is missing for the remaining part; in the other copy, the whole sequence of LKB1 (amino acids 333–340) is built but the linker region has no electron density (Figs. 1*b*, 1*c* and 1*d*).

14-3-3 ζ forms a homodimer with a twofold axis and its amino-terminal region mediates homodimerization, as seen in previously determined structures (Liu *et al.*, 1995). The root-mean-square deviations (r.m.s.d.s) of superimposition for all atoms and for C^α atoms only for apo 14-3-3 ζ (PDB entry 1a4o) and 14-3-3 ζ -LKB1 are 0.928 and 0.787 Å, respectively, suggesting that 14-3-3 ζ undergoes no major conformational changes when it binds the LKB1 peptide.

Unexpectedly, the conformations of the LKB1 peptides in the two copies of the 14-3-3 ζ -LKB1 protein are distinct from each other (Fig. 2). In one copy of 14-3-3 ζ -LKB1 the total interface area is 229.3 Å² (Figs. 3*a* and 3*b*). A sulfate ion from

the crystallization solution occupies the position where a phosphate from pS/pT should be present and forms extensive salt bridges and hydrogen bonds with Arg56 in helix α_3 and Arg127 and Tyr128 in helix α_5 from 14-3-3 ζ , which are also observed in the other copy. Arg333 in the LKB1 peptide also interacts with this sulfate ion, as well as with residue Glu180 in helix α_7 of 14-3-3 ζ , *via* polar interaction, suggesting that the phosphorylated LKB1 peptide at Thr336 contacts 14-3-3 ζ like a mode II binding motif. However, from the point of view of sequence, the LKB1 peptide resembles the mode I binding motif.

In the other copy of 14-3-3 ζ -LKB1 the total buried area is 508 Å² (Figs. 3*a* and 3*c*). The LKB1 peptide adopts an unusual conformation to interact with 14-3-3 ζ . Met335, Val338, Pro339 and Tyr340 of LKB1 are embedded into and contact the hydrophobic part of the 14-3-3 ζ amphipathic binding groove formed by Phe117 and Lys120 in helix α_5 , Pro165, Ile166 and Leu172 in helix α_7 and Leu216, Ile217 and Leu220 in helix α_9 . In addition, the main chains of residues Glu336, Val338, Pro339 and Tyr340 in LKB1 form hydrogen bonds to the side chains of residues Ser45, Lys49 and Asn50 in helix α_3 and Lys120 in helix α_5 from 14-3-3 ζ to further stabilize their association. However, the phosphomimetic residue Glu336 of LKB1 does not contact residues Arg56, Arg127 and Tyr128 in 14-3-3 ζ , which coordinate the phosphate of phosphorylated residues, but forms hydrogen bonds to Asn50 in 14-3-3 ζ . Thus, the binding of the LKB1 peptide to 14-3-3 ζ in this copy is independent of its phosphorylation at Thr336.

We compared the second conformation of the LKB1 peptide with those of other 14-3-3 ligands possessing canonical

Figure 1
(*a*) Overall structure of the 14-3-3 ζ -LKB1 fusion protein. The two molecules of 14-3-3 ζ are coloured green and cyan and are shown in cartoon representation. The LKB1 peptides are coloured slate and are shown in stick representation. (*b*) The LKB1 peptides shown along with a superimposed $mF_o - DF_c$ OMIT map (red mesh) contoured at 3.0 σ . (*c*, *d*) The $2mF_o - DF_c$ map (grey mesh) for LKB1 peptides and part of 14-3-3 ζ at 2.9 Å resolution contoured at 1.0 σ .

phosphorylation-dependent binding modes including modes I, II and III (PDB entries 1qja, 1qjb and 1o9d; Rittinger *et al.*, 1999; Würtele *et al.*, 2003), and found that the interacting regions of LKB1 and other ligands on 14-3-3 are distinct.

LKB1 mainly associates with the hydrophobic region of 14-3-3 *via* hydrophobic interactions, while the canonical binding motifs interact with the polar region of 14-3-3 mainly *via* its pS/pT (Fig. 4a).

Figure 2

Comparison of the two conformations of the LKB1 peptide in the 14-3-3 ζ -LKB1 structure. The two molecules of 14-3-3 ζ are coloured green and cyan, respectively, and are shown in cartoon representation. The LKB1 peptides in the first and second conformations are coloured slate and magenta, respectively, and are shown in stick representation.

Figure 3

(a) Structure of the 14-3-3 ζ -LKB1 fusion protein. The two molecules of 14-3-3 ζ are coloured green and cyan, respectively, and shown as a surface. The LKB1 peptides are coloured slate and shown in stick representation. (b, c) Detailed interface of the 14-3-3 ζ and LKB1 peptides. Salt bridges and hydrogen bonds are shown as magenta dashed lines.

Figure 4
(a) Structural comparison of 14-3-3 in complex with LKB1 and 14-3-3 ligands possessing mode I, II or III motifs. (b) Structural comparison of 14-3-3 in complex with LKB1, R18, ExoS, PMA2 and ChREBP peptides. (c, d, e) Detailed interactions of 14-3-3 with LKB1 (c), R18 (d) and ExoS (e). Note that the following structures from the PDB are used: 1qja, 1qjb, 1o9d, 1a38, 2o02, 2o98 and 4gnt.

Several structures of 14-3-3 in complex with unphosphorylated ligands including R18, exoenzyme S (ExoS), plasma-membrane H^+ -ATPase (PMA2) and carbohydrate-responsive element-binding protein (ChREBP) have been determined (PDB entries 1a38, 2o02, 2o98 and 4gnt; Yang *et al.*, 2006; Ottmann, Marco *et al.*, 2007; Ottmann, Yasmin *et al.*, 2007; Petosa *et al.*, 1998; Fig. 4b). Compared with the conformations of these ligands when they bind 14-3-3, the second conformation of LKB1 is different from any of these known conformations, indicating that the interaction of 14-3-3 ζ with the LKB1 peptide in the second copy represents a novel partner-binding mode of 14-3-3. When analyzing these structures in depth, we found that R18 and ExoS also occupy the LKB1-binding hydrophobic pocket of 14-3-3. In the LKB1–14-3-3 complex Pro339 and Tyr340 contact this region of 14-3-3, while in the R18–14-3-3 and ExoS–14-3-3 complexes Leu2 of R18 and Leu422, Ala425 and Leu426 of ExoS extend into this hydrophobic region, indicating that this hydrophobic region of 14-3-3 proteins plays an important role in ligand binding (Figs. 4c, 4d and 4e).

Previously, Bai and coworkers reported that LKB1 phosphorylated at Thr336 interacts with 14-3-3 proteins (Bai *et al.*, 2012). We found that LKB1 could also bind 14-3-3 ζ in a phosphorylation-independent manner. Taken together, LKB1

can interact with 14-3-3 ζ in two fashions: phosphorylated LKB1 may prefer to adopt the first canonical conformation to interact with 14-3-3 ζ , while unphosphorylated LKB1 utilizes the second noncanonical conformation. The function of the two 14-3-3 binding modes of LKB1 needs to be further investigated.

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